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Characterization of dimeric ATP synthase and cristae membrane ultrastructure from *Saccharomyces* and *Polytomella* mitochondria

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Abstract There is increasing evidence now that F_1F_0 ATP synthase is arranged in dimers in the inner mitochondrial membrane of several organisms. The dimers are also considered to be the building blocks of oligomers. It was recently found that the monomers in beef and the alga *Polytomella* ATP synthase dimer make an angle of $\sim 40^\circ$ and $\sim 70^\circ$, respectively. This arrangement is considered to induce a strong local bending of the membrane. To further understand the packing of dimers into oligomers we performed an electron microscopy analysis of ATP synthase dimers purified from *Saccharomyces cerevisiae*. Two types of dimers were found in which the angle between the monomers is either $\sim 90^\circ$ or $\sim 35^\circ$. According to our interpretation, the wide-angle dimers ($70\text{--}90^\circ$) are “true-dimers” whereas the small-angle dimers ($35\text{--}40^\circ$) rather are “pseudo-dimers”, which represent breakdown products of two adjacent true dimers in the oligomer. Ultrathin sectioning of intact *Polytomella* mitochondria indicates that the inner mitochondrial or cristae membrane is folded into lamellae and tubuli. Oligomers of ATP synthase can arrange in a helical fashion in tubular-shaped cristae membranes. These results strongly support the hypothesized role of ATP synthase oligomers in structural determination of the mitochondrial inner membrane.

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Keywords: ATP synthase; Dimer; Electron microscopy; Mitochondria; *Saccharomyces cerevisiae*; *Polytomella*

1. Introduction

The proton F_1F_0 ATP synthase is a ubiquitous enzyme that is found in virtually all living organisms. It is a multi-subunit complex of about 600 kDa involved in rotary catalysis. In prokaryotes the enzyme consists of an F_0 intrinsic membrane domain with three different subunits named a, b and c and an extramembranous F_1 domain (or headpiece), including a central stalk, with five different subunits α , β , γ , δ and ϵ . Three α and β subunits shape the F_1 headpiece; the γ , and ϵ are present in single copies in the central stalk. Most of the F_0 domain is formed by a multimer of 10 c subunits. The c-subunit multimer of the F_0 domain and the $\alpha_3\beta_3$ F_1 headpiece are linked

by the central stalk and additionally by a peripheral stalk composed of two b subunits. The peripheral stalk is connected to the top of the $\alpha_3\beta_3$ F_1 headpiece via the δ subunit. During catalysis a proton membrane gradient drives the rotation of the ring of multiple copies of the c subunit [1]. This results in a clockwise rotation of the γ subunit of the F_1 -ATPase and in the synthesis of ATP.

Mitochondrial F_1F_0 ATP synthases contain the same eight subunits, though some have a different name such as the OSCP subunit, which is the counterpart of the prokaryotic δ subunit. There are at least eight small subunits in addition to those of the prokaryotic enzyme [2]. In *Saccharomyces cerevisiae* there is one additional subunit located in the central stalk (the mitochondrial specific ϵ subunit). Most of the remaining ones are located in the F_0 domain (d, e, f, g, h, i and k) where they are positioned close to the peripheral stalk. Atomic resolution structures have revealed the folding of the α , β , γ , δ and ϵ subunits of the F_1 domain [3,4] and based on biochemical studies there is a recent lower resolution model for the arrangement of the four subunits consisting the peripheral stalk or stator in beef [5]. There is, however, no detailed structural information of the many (small) subunits of bovine or yeast ATP synthase, which form the F_0 domain outside the subunit c multimer.

Although the proton ATP synthase is catalytically active in its monomeric form a dimeric ATP synthase supercomplex from yeast mitochondria has been characterized by BN/SDS PAGE [6] and other techniques. This supercomplex includes the above-mentioned small subunits f, i, e, g, and k, the latter three of which are thought to be involved in dimer formation. Recent data show that yeast cells deficient in the dimer-specific subunits e or g lack dimeric ATP synthase [7,8]. Some data also point to an involvement of one of the subunits of the peripheral stalk, subunit b, in dimer formation [9]. Similar dimeric ATP synthase supercomplexes were found in a wide range of organisms such as beef [10], *Arabidopsis* [11], *Chlamydomonas* [12] and *Polytomella* [13,14].

Several biochemical studies indicate in addition to the dimeric conformation a further packing of ATP synthase into oligomers. The oligomerization of the ATP synthase has been proposed to determine mitochondrial morphogenesis [7,8,14]. Yeast mutants that lack the ATP synthase subunits e or g show a different type of folding of the inner mitochondrial or cristae membrane. This membrane is heavily folded, but mutants show an onion-like packing of cristae membranes. A GTPase named Mgm1p serves as a regulator of subunit e stability, ATP synthase assembly and cristae morphology [15]. The only

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direct evidence for ATP synthase oligomers was obtained in an electron microscopy study by using rapid-frozen deep-etched mitochondria from the unicellular freshwater organism *Paramecium* [16]. One of the best ways to study subcellular structures by electron microscopy is to embed them without chemical fixation in amorphous ice and to perform a three-dimensional reconstruction by electron tomography [17,18]. But for relatively large objects like mitochondria it remains particularly difficult to obtain a resolution sufficient to see the packing of individual copies of the major protein complexes like the ATP synthases, because the attainable resolution is directly related the size of the object [19]. In the best current available reconstructions the F_1 headpieces come into focus, but they do not show how the oxidative phosphorylation complexes are precisely organized [17].

Until now a low-resolution structural characterization of the ATP synthase dimer has been carried out in parallel for two different species: beef [20] and the colorless alga *Polytomella* [14]. In both cases it was found that the two F_1F_0 monomers are making an unexpected angle which was $\sim 40^\circ$ in the case of beef and $\sim 70^\circ$ for *Polytomella*. Because of this angle the membrane around the F_0 parts of the dimer is curved. It was concluded that this kink drives the mitochondrial cristae membrane to adopt a local curvature [14,20]. A major difference between the two dimers, however, is a difference of $\sim 30^\circ$ between the two angles. This discrepancy may be explained by the fact that the two species are not closely related and have a different subunit composition. But it may also be possible that the association of monomers in the dimers is different. To get further insight into the dimer configuration we performed single particle electron microscopy analysis on a third species, the yeast *S. cerevisiae*. Since *Polytomella* ATP synthase dimers were found to be unusually stable [14], additional ultrathin sectioning of these mitochondria was performed to investigate the overall membrane packing of the oxidative phosphorylation complexes. The analysis of the yeast dimers indicates that detergent solubilization of mitochondrial membranes leads actually to two distinct types of dimers, either with an angle of about 90° between the long axes of the F_1F_0 monomers or with an angle of 35° . Both types of dimers are considered to originate from an oligomeric organization of ATP synthases in rows. Ultrathin-sectioning and negative staining of osmotically shocked *Polytomella* mitochondria show that such oligomers are (partly) arranged in a helical fashion in tubular-shaped cristae membranes.

2. Materials and methods

2.1. Cultivation of *Polytomella* spp. and *Saccharomyces cerevisiae*

Polytomella spp. (198.80, E.G. Pringsheim) was obtained from the “Sammlung von Algenkulturen der Universität Göttingen” (Germany) and cultivated as described in Dudkina et al. [14]. *S. cerevisiae* (strain Y187) was cultivated in YPD medium. For mitochondrial isolations, cells were transferred into lactate medium (2.6 mM glucose, 7.3 mM KH_2PO_4 , 18.7 mM NH_4Cl , 4.5 mM $CaCl_2$, 8.6 mM $NaCl$, 2.9 mM $MgCl$, 2.2% lactate).

2.2. Preparation of mitochondria

Mitochondria of *Polytomella* were isolated by differential centrifugation and Percoll density gradient ultracentrifugation as outlined previously [14]. Isolation of yeast mitochondria was based on differential centrifugations and sucrose gradient ultracentrifugation as described by Meissinger et al. [21]. Mitochondria were shock-frozen using liquid nitrogen and stored at $-80^\circ C$ until use.

2.3. Purification of dimeric ATP synthase

The membrane-bound protein complexes of yeast and *Polytomella* were solubilized using digitonin solution (5% detergent, 30 mM HEPES, 150 mM K-acetate, 10% glycerine, pH 7.4) and separated by sucrose gradient ultracentrifugation (gradients of 0.3–1.5 M sucrose, 15 mM Tris base, pH 7.0, 20 mM KCl, 0.2% digitonin; centrifugation for 17 h at $150,000 \times g/4^\circ C$). Afterwards the gradients were fractionated and the protein complex compositions of the fractions analyzed by 1D Blue-native PAGE [22].

2.4. Electron microscopy

Selected fractions of the gradients including dimeric ATP synthase were directly used for electron microscopy and single particle analyses [23]. For ultrathin sectioning, *Polytomella* mitochondria were isolated according to [14], pelleted and double fixed with glutaraldehyde and osmium tetroxide. Fixed mitochondria were dehydrated with acetone and embedded in Epon. Ultrathin sections were made on an ultramicrotome and double stained with uranyl acetate and lead citrate.

For studying the localization of the oxidative phosphorylation system in the inner membrane before detergent disruption *Polytomella* mitochondria were osmotically shocked by diluting them 10× in distilled water on electron microscopy grids and directly stained with uranyl acetate.

3. Results

3.1. Purification of yeast ATP synthase dimers

Solubilization of isolated yeast mitochondria by digitonin and analysis of the solubilized protein complexes by 2D Blue native/SDS PAGE allowed to monitor the known complexes of the respiratory chain (Fig. 1). In accordance with previous publications [6], ATP synthase is present in two forms, a monomeric complex of about 500 kDa and a dimeric complex of about 1000 kDa. In-between, supercomplexes of dimeric complex III and one or two copies of monomeric complex IV are visible. Furthermore, two forms of dimeric complex III are present below the monomeric ATP synthase complex, which represent the intact dimer and a subcomplex of the dimer lacking the Rieske FeS subunit and another 8.5 kDa subunit. Both subunits are known to easily get detached from the dimeric complex III [24]. The ratio of monomeric and dimeric ATP synthase very much depends on the detergent used for solubilization [6]. The dimer is best stabilized in the presence of low Triton X-100 concentrations. However, digitonin and dodecylmaltoside also proved to be suitable detergents for ATP synthase supercomplex stabilization [10].

In contrast, ATP synthase of *Polytomella* is exclusively visible in the dimeric form on 2D Blue native/SDS gels independently of the type of non-ionic detergent used for solubilization (Fig. 1, [13,14,25]). The extraordinary stability of the dimer most likely is due to an additional large subunit called “mitochondrial ATP synthase associated protein” (MA-SAP), which runs at 60 kDa on the second gel dimension (Fig. 1).

For successive EM analysis of yeast ATP synthase, Blue-native PAGE was substituted by sucrose density gradient ultracentrifugation to obtain protein complexes in solution. The gradients were fractionated and small aliquots of the fractions were analyzed by Blue-native PAGE to monitor their protein complex composition. A fraction close to the bottom of the gradients (fraction 5) included pure ATP synthase dimers. The corresponding fractions were directly used for EM and single particle analysis.

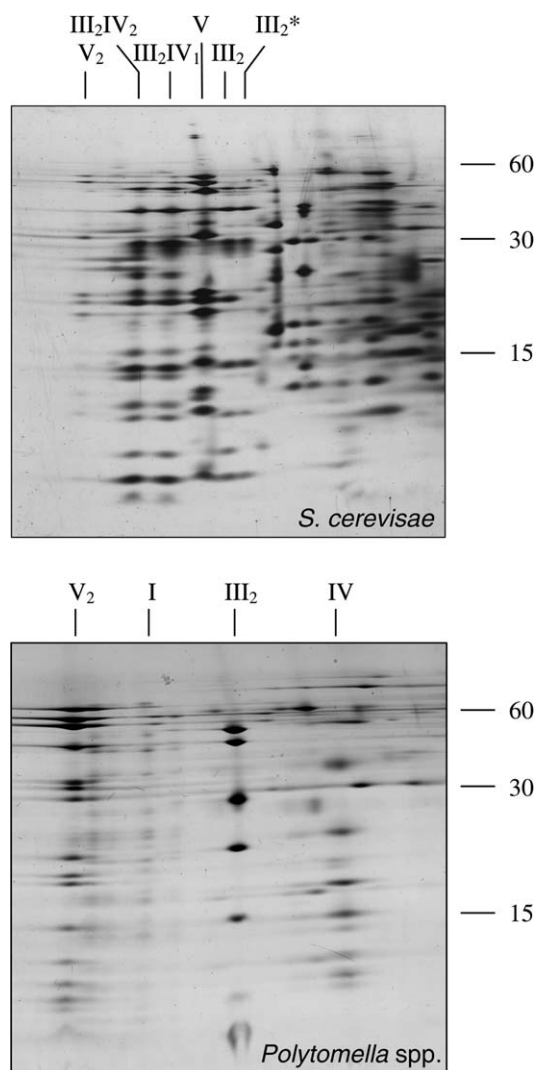


Fig. 1. Separation of OXPHOS complexes from *Saccharomyces cerevisiae* and *Polytomella* spp. by 2D Blue-native/SDS-PAGE. Mitochondrial proteins were solubilized with digitonin (5 g per g protein) as described under experimental procedures. Identities of the protein complexes are given above the gels and molecular masses of standard proteins to the right (in kDa). V_2 : dimeric ATP synthase; I: complex I; III_2 : dimeric complex III; III_2^* : subcomplex of dimeric complex III lacking the FeS subunit; III_2IV_2 : supercomplex composed of dimeric complex III and two copies of complex IV; III_2IV_1 : supercomplex composed of dimeric complex III and one copy of monomeric complex IV; IV: complex IV.

3.2. Structural analysis of yeast ATP synthase dimers by electron microscopy

Negatively stained specimens of the fractions were found to contain large numbers of dimeric ATP synthase molecules (Fig. 2). The only obvious contamination was the supercomplexes of complex III and IV, but because the latter looks quite different and because the shape of the ATP synthase dimers is very characteristic, it was possible to select a rather homogeneous data set of 20,000 single particle projections. Single particle analysis showed the presence of seven different views, representing two types of ATP synthase dimers and their apparent breakdown fragments. The main difference between the two types of dimers is in the angle between the monomers, which is either about 90° (Fig. 3(A)) or 35° (Fig. 3(E)). A smaller differ-

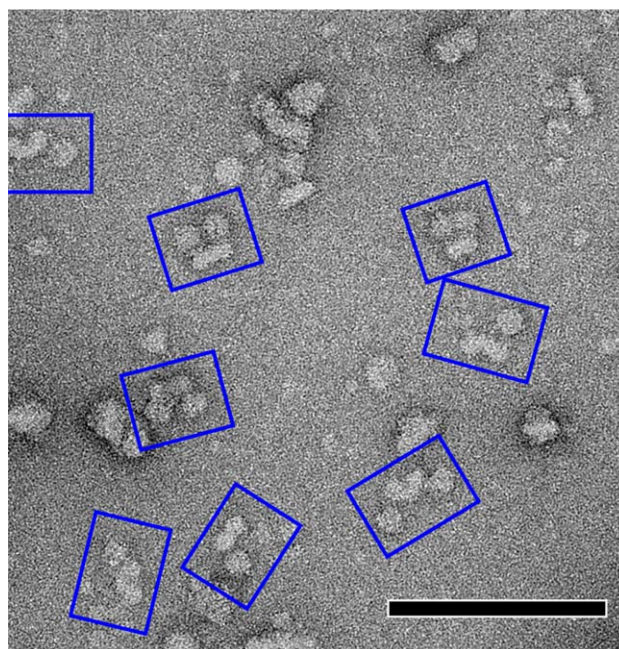


Fig. 2. Electron micrograph of membrane proteins from the fraction 5 obtained by sucrose gradient centrifugation. Blue boxes indicate ATP synthase dimers, unassigned proteins could be partly the supercomplex of complexes III and IV (see text). The scale bar is 100 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ence is in the total width of the F_0 domains in projection, which is larger in the wide-angle dimers. Both angles are found to remain in particles missing one headpiece (Fig. 3(B), (C), (F) and (G)) and even both headpieces (Fig. 3(D)). The ratio between the two types of dimers, including their fragments, is about 1:1.

The peripheral stalk of the yeast dimer appears faint (white arrows; left headpieces in Fig. 3(A) and (B)) or is absent (all other headpieces). This is a striking difference with the previously analyzed *Polytomella* dimer (Fig. 3(H)). Another difference is at the top of the F_1 headpiece because the yeast F_1 headpiece is clearly seen to be blunt in all views. In the best-resolved headpiece of the particle of Fig. 3(C) there is a stain accumulation at the site of the tip (white arrowhead) at where the OSCP subunit should be present. The absence of (most of) the OSCP subunit explains the absence of the stator because the stator needs OSCP for binding to the headpiece. Despite the large number of particles analyzed the resolution was low in comparison to *Polytomella* and the best resolution of about 30 Å was obtained for one of the fragments (Fig. 3(C)). This low resolution is likely due to flexibilities in the dimer such as small angular deviations between two monomers or a movement between the F_0 and F_1 domains because of the absence of the stator in most monomers. A further difference with *Polytomella* is in the membrane part; in yeast dimers the width is somewhat larger (Fig. 3(A)) or smaller (Fig. 3(B)) than in those of *Polytomella* (Fig. 3(H)).

3.3. Cristae morphology

Electron microscopy analysis of thin-sectioned *Polytomella* mitochondria shows it is a spherical organelle of approximately 3 µm in diameter (Fig. 4(A)). Two types of cristae membranes could be observed in these mitochondria. There are lamellae

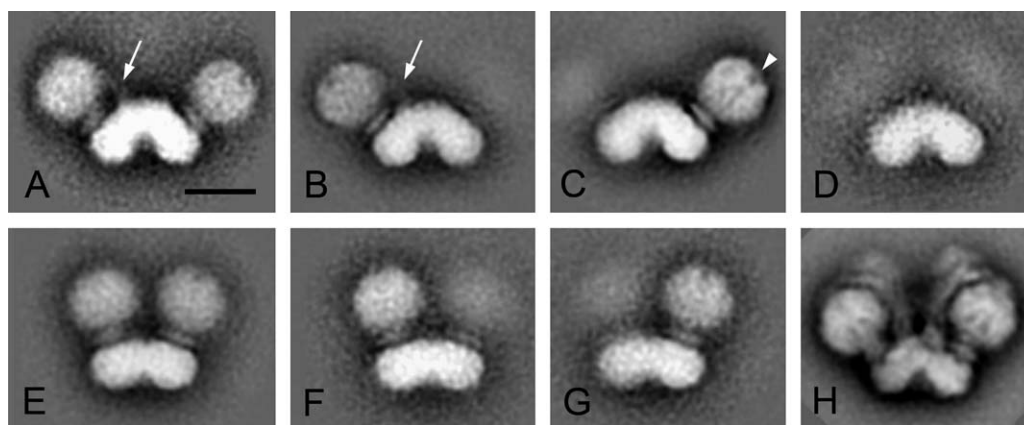


Fig. 3. Projection maps of purified dimeric ATP synthase complexes from *S. cerevisiae* (A–G) obtained by single particle averaging. A, E – complete dimers; B, C, F, G – dimeric ATP synthase fragments lacking one F_1 headpiece; D – fragment of supercomplex lacking two F_1 headpieces; H – complete ATP synthase dimer from *Polytomella* (from Dudkina et al. 2005). White arrows indicate the stator at the right side of the left headpiece; white arrowhead indicates the absence of the OSCP subunit. The bar is 10 nm.

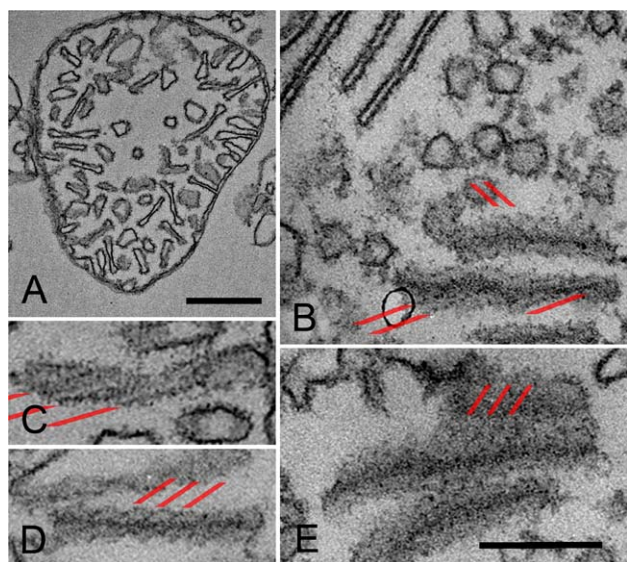


Fig. 4. Electron microscopy of ultrathin-sectioned *Polytomella* mitochondria. (A) Overview of a mitochondrion. The bar is 1 μ m. (B–E) Selected higher-magnification parts of thin-sections showing lamellae (B) and mostly tubular cristae membranes with some periodicity in the arrangement of protein complexes (B–E). The red bars indicate the angle between the periodic ordering and the long axis of the tubules. The bar for frames B–E is 500 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with a spacing of about 25 nm between the membranes (upper left corner, Fig. 4(B)) and tubes with a diameter of 60 nm or more. In several of the tubes that run parallel to the sectioning direction a kind periodicity in the arrangement of protein complexes is visible (red bars, Fig. 4(B–E)). Although individual complexes are not well visible it appears that this ordering of protein complexes makes an angle with the long axis of the tubules, as indicated by the red bars. Because the resolution in the sections was low we also applied negative staining with uranyl acetate on osmotically shocked mitochondria. Similar rods with a diameter of 60–90 nm are present in osmotically shocked mitochondria (Fig. 5). In some of the tubules the ATP synthase headpieces are well resolved (upper left frame, Fig. 5), but no

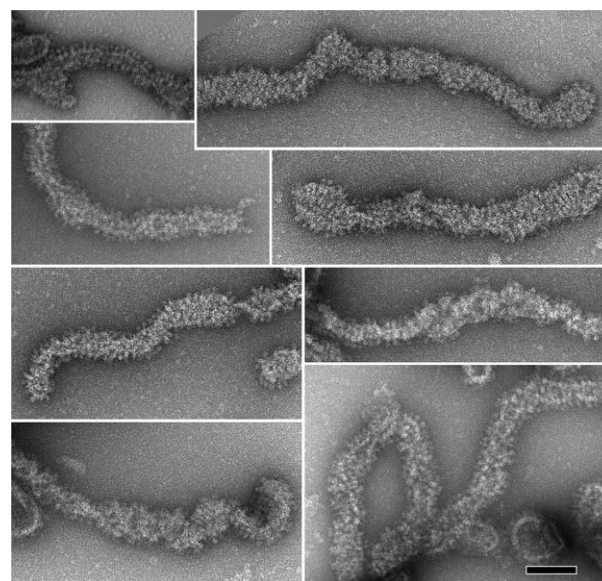


Fig. 5. Negatively stained mitochondrial tubular cristae fragments of *Polytomella*. The largest protruding densities are ATP synthase headpieces, which are especially dominant in the upper left electron micrograph. The space bar for all frames is 100 nm.

visible ordering of proteins seems to be preserved. The fact that the observed ordering makes an angle with the long axis of the tubules from the thin sections is typical for a helical ordering of protein complexes. Since the complexes were metal stained, the stronger stained dark zones should represent ATP synthase complexes, because the ATP synthase headpieces protrude at least two times farther away from the membrane as any of the complexes I–IV. Hence, the tubules seen in the sectioned *Polytomella* mitochondria are interpreted as composed of helically arranged rows of ATP synthase complexes separated by zones occupied by the respiratory complexes I–IV.

4. Discussion

In this report, we present an analysis of ATP synthase dimers isolated from *S. cerevisiae* mitochondria by electron

microscopy and single particle image analysis in combination with the ultrathin sectioning and negative staining of the whole mitochondria from the alga *Polytomella*. The aim of this analysis is the detection of the way in which ATP synthase dimers/oligomers are organized in the mitochondrial inner membrane. We analyzed the projection structure of the yeast ATP synthase dimer in which complete dimers and fragments missing one or both of the F_1 parts clearly indicate that the interaction between the monomers occurs by their F_0 parts. The monomers make an angle between of either about 90° or 35° , but other angles were not observed (Fig. 3). In *Polytomella* there appears to be only one type of ATP synthase dimer (Fig. 3(H), [14]), in which the angle is about 70° . This causes the membrane-embedded F_0 parts to have a different orientation, but otherwise they look quite similar. In the case of the bovine ATP synthase there are only dimers with a small (40°) angle between the monomers [20], quite similar to the 35° angle observed in yeast particles (Fig. 3(E–G)). Both small angle dimers show the absence of stators in projection, which could mean that they are not visible because of an overlap-situation with the headpieces. But because the large angle yeast particles appear to lack most of the stators, it could well be that the same is true for the small angle dimers. The loss of stators might be caused by the low abundance of the OSCP subunit. It is possible that this subunit is partly lost during EM specimen preparation due to the high (5%) digitonin concentration or more likely a lower stability of the yeast ATP synthase dimers. However, the absence or presence of the stator and the OSCP subunit do not appear to have a major effect on the overall shape of the dimers, because even in the absence of one or two complete F_1 headpieces the angle between the F_0 parts is maintained.

The question now arises what makes the ATP synthase dimer to appear seemingly in two conformations. Until now several studies, as well as our results (see below), point to oligomerization of ATP synthases and thus dimeric supercomplexes are thought to be the building blocks of ATP synthase oligomers. According to our scheme (Fig. 6), we propose that two types of dimers appear as breakdown products of oligo-

mers after detergent solubilization of cristae membranes. This could lead to “true-dimers” with an observed angle of 70° or 90° and to “pseudo-dimers” with angle of 35° or 40° consisting of two neighbour monomers from two broken native dimers, as depicted in the scheme. The fact that the two types of dimers appear rather than a change in the angular position of the monomers is clear from the width of the F_0 parts. The F_0 part from the true-dimers (Fig. 3(A)) is about 40% wider than in the pseudo-dimers (Fig. 3(E)). The fact that the pseudo-dimers were not observed in *Polytomella* could have to do with the fact that the intra-dimeric interaction in the ATP synthase oligomers is much stronger than the inter-dimeric interaction. It would also be worthwhile to re-examine the beef dimers to see if any type of wide-angle dimers is present at low frequency. Finally, a lower intra-dimeric interaction could correlate with the low abundance of intact stators in yeast (Fig. 3) and bovine dimers [14,20] and the high abundance in *Polytomella* [14], where the stator is very pronounced by the presence of MASAP protein in its upper part. Biochemical data also indicate that dimer specific subunits of yeast are attached to the stator close to the F_0 part [2].

Supporting evidence for the scheme of Fig. 6 is presented in a BN-PAGE analysis of mammalian mitochondria which resulted in several types of ATP synthase multimers [26]. About 30–35% of total ATP synthase was obtained as three low-mobility species with apparent molecular masses of ~ 1500 , ~ 2300 , and ~ 2900 kDa, and the remainder as monomeric ATP synthase (~ 750 kDa). These low-mobility species represented dimeric ATP synthase, as well as trimeric and tetrameric ATP synthase [26]. The trimeric ATP synthase fragments can now be explained as an expected breakdown product if the strength of inter- and intra-dimeric interactions are about equal. The position of the small mitochondrial ATP synthase subunits b, e, f, g, i and k around the dimer interface is not precisely known. It is not yet possible to propose their role in intra- and interspecific dimer contacts. But recently it was proposed that subunits e and g are important in interspecific interaction [7,8]. This is a further support for the interpretation of the two different types of dimers as being different breakdown products of oligomers.

Because the best current available reconstructions [17] do not show how the oxidative phosphorylation complexes are precisely organized we used classical ultrathin sectioning to get hints about the packing of proteins in the mitochondria membrane. Sectioning of *Polytomella* mitochondria indicates a helical packing of cristae membrane proteins in tubules (Fig. 4). Because these tubular membranes are full of oxidative phosphorylation complexes as shown by additional negative staining of osmotically shocked mitochondria from *Polytomella* (Fig. 5) these data clearly hint to an oligomeric organization of ATP synthases in a helical fashion. The observation of rows of ATP synthases arranged in a helical way was previously demonstrated at higher resolution for mitochondria from the unicellular freshwater organism *Paramecium* by rapid-freeze deep-etch electron microscopy [16,27]. The latter studies indicated that ATP synthase dimerization and the cristae morphology are linked to each other. Yeast mutants devoid of dimer-specific subunits were shown unable to form dimeric ATP synthase and comprise mitochondria with drastically changed morphology [7,8]. Instead of the characteristic highly folded inner membrane architecture, the cristae, the membranes consist of atypical “onion-like” structures. Similar

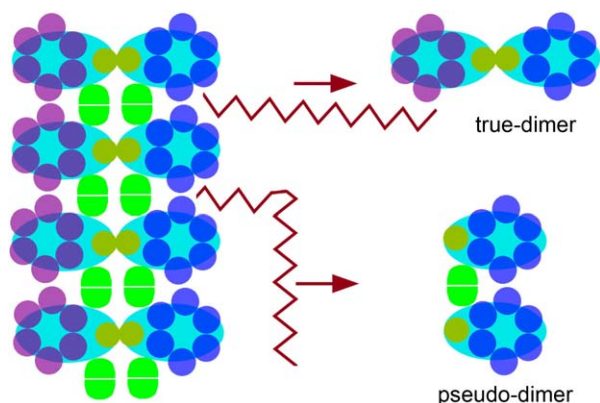


Fig. 6. A model for the arrangement of ATP synthases dimers into multimers. Oligomers consist of dimeric ATP synthases. The oligomers can break down by detergent incubation into “true-dimers” or into “pseudo-dimers”. The latter actually consist of two monomers from the neighbouring dimers, symbolized by a blue and purple set of $\alpha_3\beta_3$ subunits. Ochre and bright green densities symbolize dimer- and interdimer specific subunits, respectively.

mitochondrial morphology was observed in yeast cells containing in vivo cross-linked F_1 -headpieces [28]. The presence of a surprisingly large angle of 90° between two membrane parts of neighbouring monomers in yeasts nicely confirms the hypothesis that ATP synthase supercomplexes are essential for the inner membrane folding.

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